RESEARCH LETTER

# Grapevine xylem sap enhances biofilm development by *Xylella fastidiosa*

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## Abstract

*Xylella fastidiosa* is able to form biofilms within xylem vessels of many economically important crops. Vessel blockage is believed to be a major contributor to disease development caused by this bacterium. This report shows that *Vitis riparia* xylem sap increases growth rate and induces a characteristic biofilm architecture as compared with biofilms formed in PD2 and PW media. In addition, stable cultures could be maintained, frozen and reestablished in xylem sap. These findings are important as xylem sap provides a natural medium that facilitates the identification of virulence determinants of Pierce's disease.

## Introduction

*Xylella fastidiosa* (*Xf*) is able to colonize a vast array of plants and cause economically important diseases. This Gram-negative bacterium is xylem limited, and extensive colonization of xylem vessels is believed to be associated with leaf scorching and chlorosis symptoms commonly seen on diseased plants. Although the exact mechanism of pathogenesis is not fully understood, vessel blockage by bacterial biofilms and by plant-derived gums and tyloses seem to be necessary for disease development (reviewed in Chatterjee *et al*., 2008).

Besides vessel blockage, biofilm formation is proposed to contribute to successful colonization of plant tissues by protecting cells embedded in the matrix against plant defenses and other environmental stresses. The development of structured bacterial communities is also known to affect regulation of enzymatic functions thereby facilitating breakdown of host tissue (reviewed in Danhorn & Fuqua, 2007).

Studies on *Xf* biofilm formation under natural conditions, i.e., *in planta*, have been hindered in part by the optical inaccessibility of vascular tissue, as well as difficulties in culturing this fastidious pathogen in grapevine xylem sap. Since the development of *Xf* culture media, for example Pierce's disease #2 (PD2) and Periwinkle wilt (PW) (Davis *et al*., 1980), all *in vitro* cultural studies, with the exception of one (Andersen *et al*., 2007), have been performed with artificial media. In part, this is because xylem sap is not available in large quantities throughout the year and perhaps most importantly *Xf* could not be readily cultured in sap following culturing on artificial media. Recent studies have shown the importance of xylem fluid chemistry on growth, aggregation and attachment of *Xf* cells, highlighting the importance of establishing stable cultures in sap, or sap : PD2 mixtures (Leite *et al*., 2004; Andersen *et al*., 2007; Bi *et al*., 2007). Here it is shown that sap collected in the early spring supports a stable culture of *Xf* and strongly influences attachment and biofilm formation and that

sap : PD2 mixtures support increased growth rates. Furthermore, sap provides a natural medium that will be useful for studying *Xf* interactions with the host plants and vectors including the identification of virulence determinants.

## Materials and methods

### Collection and storage of xylem sap

Grapevine xylem fluid (sap) was collected in the spring (April, 2008) from bleeding *Vitis riparia* grapevines grown near Geneva, NY. *Vitis riparia* is known to be susceptible to Pierce's disease. Sap was collected from cut canes as it dripped into 1-L tissue culture flasks. Collections were made from 10 plants, with up to 500 mL per plant obtained over a 4-h period. The samples were pooled, passed through a glass wool filter, filter sterilized with a 0.22-μm membrane filter (cat. # 431154; Corning), aliquoted into 40-mL lots and maintained frozen at − 80 °C until used. Upon thawing, and before use, the samples were once again passed through a 0.22-μm membrane filter to further assure sterility of the medium and to remove any precipitates resulting from the freezing process.

### Strains and culture conditions

The wild-type isolate of *Xf* Temecula (ATCC 700964), originally isolated from a grapevine with Pierce's disease grown in Temecula, CA (Van Sluys *et al.*, 2003), was maintained at 28 °C on PW (Davis *et al.*, 1981) agar modified by omitting phenol red, and by adding 12.5 mL $L^{-1}$ of bovine serum albumin fraction V solution (cat. # 15260; Gibco) (Galvani *et al.*, 2007). Isolates were stored at − 80 °C in modified PW broth or *V. riparia* xylem sap containing 20% glycerol.

*Xf* was also grown in liquid PD2 media (Davis *et al.*, 1981), in PD2 diluted with xylem sap, or in xylem sap alone. Glass tubes (13 × 100 mm) containing 3 mL of media were seeded with *Xf* to an initial $OD_{600\,nm}$ of 0.05, incubated at 28 °C, and shaken at 200 r.p.m. Growth of *Xf* in 100% sap was achieved following acclimation of the pathogen through an ascending series of PD2 : sap concentrations, – 100 : 0, 50 : 50, 20 : 80, 10 : 90, 0 : 100 – with sequential transfers (passages) made every 7 days.

### Growth rate analysis

Cell growth was assessed over a period of 10-days in glass tubes as described above from which growth curves were constructed. Cells attached to tube inner walls were dispersed using an inoculating loop followed by repetitive pipetting to disrupt cell clumps. Turbidity was immediately measured at $OD_{600\,nm}$ to minimize possible effects of cell aggregation. The experiment was repeated five times. Fifty microliters of the inocula used in each passage were plated onto PW medium to ensure culture purity throughout the experiment.

### Biofilm assessment

Biofilm development and morphology was assessed in the various PD2 : sap mixtures on glass slides. Slides were affixed to the inner surface of the wide-mouthed glass jars (cat. # 02-911-420; Fisher Scientific) with polydimethylsiloxane (Sylgard 184). Following sterilization by autoclaving, 20 mL of the PD2 and/or sap media were added to the jars, followed by seeding with *Xf* to an initial $OD_{600\,nm}$ of 0.05, and incubation at 28 °C with agitation at 100 r.p.m. for up to 10 days. Subsequently, the glass slides were removed, cleaned of extraneous cells on the backside and the biofilms imaged by scanning with an Epson Perfection 4870 Photo scanner at 600 dpi resolution and managed with ADOBE PHOTOSHOP. For scanning electron microscopy (SEM), the same set-up was used with the addition of coverslips attached to the glass slides with a small quantity of polydimethylsiloxane. Once removed from the jar and then from the glass slides, coverslips were prepared for SEM according to Meng *et al.* (2005).

Biofilm development was also assessed in 96-well polystyrene culture plates. *Xf* cells suspensions were made to an initial $OD_{600\,nm}$ of 0.1 in various PD2 : sap mixtures and 150 μL were added to wells of 96-well polystyrene plates (Falcon 35-1177; Becton Dickinson). Cultures were maintained for 5 days at 28 °C with agitation at 200 r.p.m. A Synergy 2 plate reader (Biotek) was used to quantify the $OD_{600\,nm}$ in each well, and this was considered the 'total' measurement. One hundred microliters of the supernatant of each well was transferred to wells in new plates and once again absorbance measured. This was considered the 'planktonic cell' measurement. Original plates were then rinsed three times with water using a plate washer (Biotek). This step removes planktonic cells and any cells loosely attached or deposited in the bottom of the well. To determine attached cells, 200 μL of aqueous 0.1% crystal violet was added to each well and plates kept at room temperature (*c.* 23 °C) for 20 min. Plates were washed three times as before with water, followed by the addition of 200 μL of 6 : 4 acetone : ethanol and agitated for 5 min. The acetone : ethanol–dye solution was measured at $OD_{600\,nm}$. This was considered the 'attached cell' measurement. A total of 44 samples for each media condition were used. Ten microliters from the 'planktonic' suspensions were serial diluted and 10 μL of each dilution dropped onto PW agar plates without spreading to determine the number of CFU. Independent dilutions were prepared with samples from three 96-well plates and the number of colonies for each condition was averaged.

## Results

Given the difficulties encountered in previous attempts to culture *Xf* in sap, mixtures containing xylem sap and PD2 medium (1 : 1) were initially used and at weekly intervals the concentration of sap was increased until cells were growing well in 100% sap. Following this acclimation procedure, a stable culture could be maintained, stored frozen and used to start new cultures with 100% sap. The frozen cells were also viable when plated on PW agar medium, the semi-solid medium routinely used in our laboratory, or liquid PD2. Subsequently, it was also verified that cells would grow at lower growth rates if transferred directly from PW plates or liquid PD2 directly to sap.

Growth of *Xf* in different PD2 : sap mixtures is shown in Fig. 1. The growth curves showed a consistent boost in cell number when supplemented with sap at all concentrations. Stationary phase was reached faster (4 days) when grown in 100% sap, possibly due to nutrient limitation. The mixtures containing between 50% and 90% sap supported increased growth beyond those observed for either 100% sap or 100% PD2. In addition to differences in growth rates, attachment to the glass surface was also greater in the presence of sap (Fig. 2). The resulting biofilm was also more strongly attached to polystyrene surfaces, especially in 90% sap, as assayed by crystal violet staining, because the washing step in the procedure removes loose cell aggregates deposited on the bottom of the well and cells loosely attached to the wall (Fig. 3a). These cell aggregates influence the 'total' OD of the tube (Fig. 3a). Such aggregates can be seen in Fig. 2. Besides being attached to surfaces or deposited as aggregates on the bottom of the wells, cells can also remain planktonic (Fig. 3b). The number of CFU in the planktonic suspensions correlated well with the $OD_{600\,nm}$ ($R^2 = 0.9323$) in both PD2 and xylem sap, suggesting that planktonic cell viability was not affected by sap. A comparison of the subpopulations of attached and planktonic cells shows that they are equivalent when grown in PD2 whereas there are significantly more

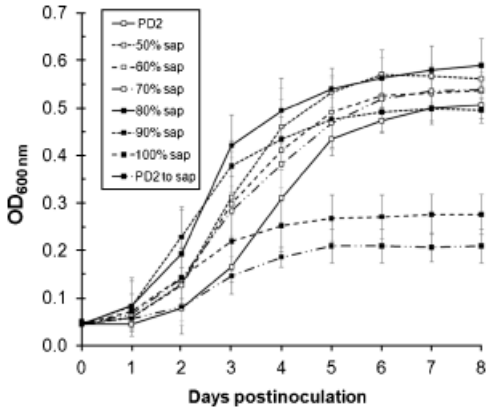


**Fig. 1.** Growth of *Xylella fastidiosa* in mixtures of PD2 and grape xylem sap. Following acclimation of cells to sap (see text), cell densities in PD2-sap mixtures were measured daily at 600 nm. The medium containing 80% sap supported the greatest growth. Average and SD of five independent experiments are shown.

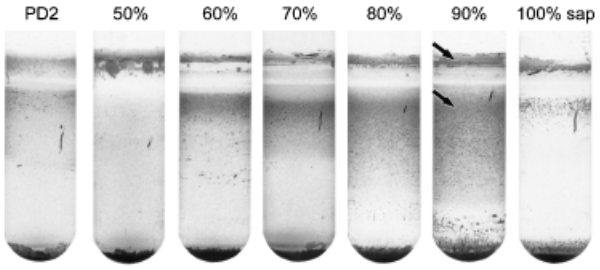


**Fig. 2.** Formation of biofilms on glass surfaces by *Xylella fastidiosa*. Cells were cultured at 28 °C and 200 r.p.m. and photographs taken after 7 days. Arrows indicate the biofilm formed at the air–liquid interface and submerged regions. Highest growth and more robust biofilms are attained at high sap : PD2 ratios.

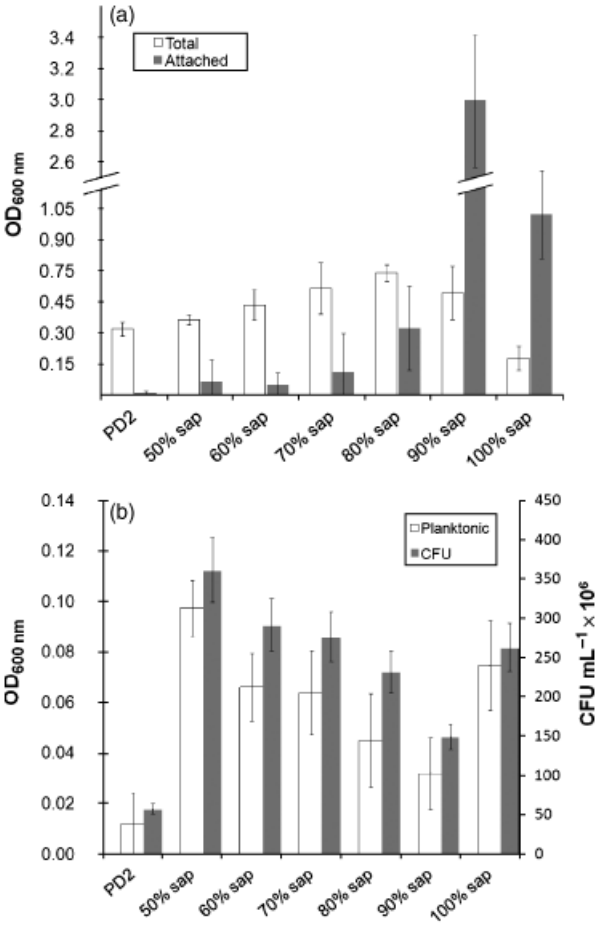


**Fig. 3.** Effect of xylem sap on growth and biofilm formation on polystyrene surfaces by *Xylella fastidiosa*. (a) After 4 days of growth at 28 °C and 200 r.p.m. in 96-well plates, OD of total and attached cells were measured. (b) OD of planktonic cells were also measured and numbers of CFU counted.

attached cells for media amended with sap and particularly at concentrations >80% (Fig. 4).

Given the difference in biofilm macrostructure observed on tube walls when cells were cultured in all concentrations of sap, more detailed observations were made using microscope slides affixed inside jars as substrata for biofilm formation. As in the glass tubes, not only did the biofilm develop sooner but the architecture was different when grown in sap compared with PD2 (Fig. 5a). Xylem sap induces the formation of a denser biofilm at the air–liquid interface. While the biofilm formed in PD2 covers most of the slide surface and is only a few cell layers in depth, the biofilm formed in media composed of sap displays secondary structure many cell layers thick (Fig. 5b).

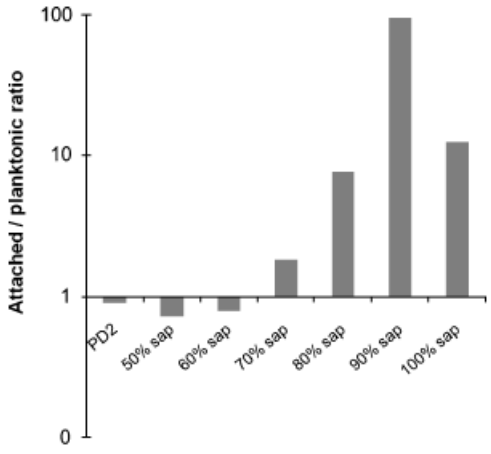


**Fig. 4.** Comparison of subpopulation numbers of attached and planktonic cells of *Xylella fastidiosa*. Obtained from data on Fig. 3.

## Discussion

Both complex and defined culture media have been developed for *Xf* enabling routine culturing and storage in laboratory conditions (Davis *et al.*, 1981; Chang & Donaldson, 1993; Lemos *et al.*, 2003; Leite *et al.*, 2004). Only recently have *in vitro* studies been reported using xylem sap to either culture *Xf* or to examine aggregation and attachment (Leite *et al.*, 2004; Andersen *et al.*, 2007; Bi *et al.*, 2007), and only one of these studies (Andersen *et al.*, 2007) reported growth in 100% sap. This lack of culturing in sap may reflect the often failure of *Xf* to survive following transfer from a nutrient-rich artificial media directly to nutritionally poor sap. Maintaining *Xf* in artificial media over several passages has been reported to influence gene expression including attenuation of virulence factors (de Souza *et al.*, 2003, 2004), at least for the citrus-infecting strain 9a5c. Whether such changes in gene expression occur following continuous growth in sap will need to be determined.

Previous work has shown that supplementing PD3 media with xylem sap, to concentrations of 25% and 50% sap, resulted in less biofilm formation when compared with PD3 alone (Bi *et al.*, 2007). The present report shows that supplementation of PD2 media with sap from *V. riparia* results in more biofilm formation at all concentrations tested (50% and up), with a peak around 70–90% sap for

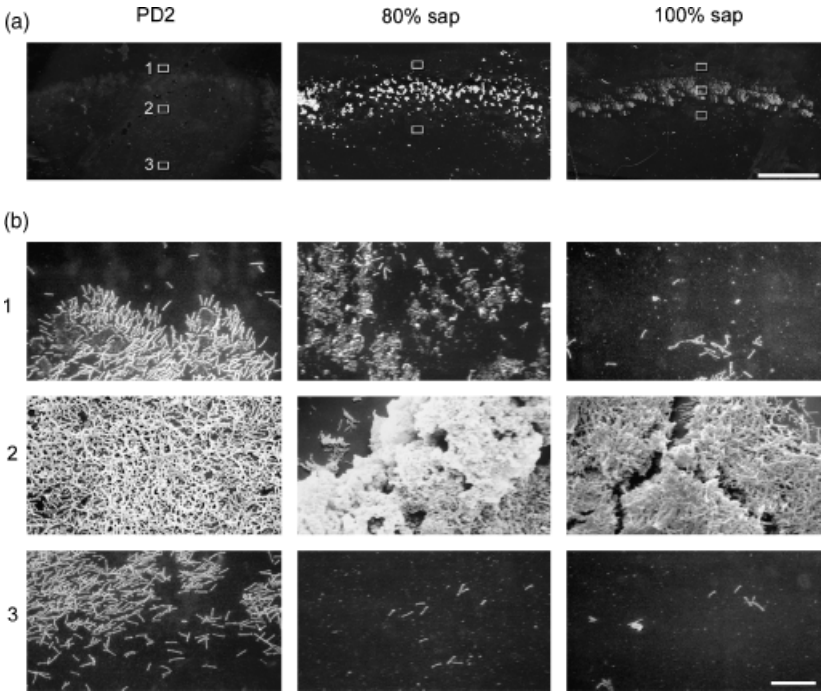


**Fig. 5.** Effect of xylem sap on morphology and structure of biofilms formed by *Xylella fastidiosa* on glass surfaces. (a) Scanner images of biofilm formed after 3 days growth in PD2, 80% and 100% sap. Numbers indicate regions of the top (air–liquid interface), middle and bottom of the cover slips shown in detail in (b). Scale bar = 0.5 cm. (b) SEM images of regions highlighted in (a). Note the effect of sap resulting in a more compact biofilm, narrower in length (*Y*-plane) and thicker in height (*Z*-plane). Scale bar = 10 μm.

glass surfaces (Fig. 2) and 80–100% sap for polystyrene surfaces (Fig. 3a). Comparing with PD2, the lower planktonic cell titer at 90% sap and the much higher attached-to-planktonic ratio (Fig. 4) further indicates the effect of xylem sap from *V. riparia* on increasing the attachment of *Xf* cells to polystyrene surfaces. As noted by Andersen *et al.* (2007), exposure of *Xf* to xylem fluids collected from different *Vitis* genotypes resulted in highly significant differences in both planktonic growth and biofilm formation. Although 100% sap did not support planktonic growth as well as PD2 beyond a few days, the initial growth rate (Fig. 1) and overall biofilm formation (Fig. 3a) was higher in sap than in PD2. It is interesting to note that under the influence of sap mound and dome-like structures several cell layers thick are formed (Fig. 5), which were not seen with PD2 media. Such structures are believed to comprise a colony structure that facilitates optimal mass transport and nutrient exchange between the bulk fluid and the cells in the biofilm than would a confluent biofilm (de Beer *et al.*, 1994).

Further experimentation will be necessary to evaluate the possible advantages to *Xf* conferred by this increase in biofilm formation when exposed to xylem sap. Among the possibilities is, for example, increased resistance to certain environmental stresses as well as antimicrobial tolerance, consortial metabolism or the opportunity for horizontal gene transfer, as has been described in other bacterial species (Sørensen *et al.*, 2005). The high population density provides the opportunity to perform certain processes that single cells cannot accomplish efficiently, such as the production of excreted metabolites or exoenzymes that are only effective above a threshold concentration (Danhorn & Fuqua, 2007). In addition, the biofilm exopolymer can also play a role in accumulating more efficiently nutrients and signals (Wolfaardt *et al.*, 1994; Liu *et al.*, 2007). Considering this, growing *Xf* in sap is a step forward studying in the biology of this pathogen in a natural medium. For example, it is known that bacterial biofilm architecture can be widely influenced by components of artificial media such as carbon sources (Breugelmans *et al.*, 2008). Culturing *Xf* in sap will also be important for research on interaction with insect vectors because cells grown on rich solid media are acquired by vectors through artificial feeding systems but not transmitted to plants (R.P.P. Almeida, unpublished data).

Whether the differences in biofilm formation observed are due to the chemical or physical environment in sap remains unknown. A recent study showed the influence of pH in aggregate formation by *Xf* (Wulff *et al.*, 2008). At least in the case of PD2, lowering the pH to that of xylem sap used in this study (pH 5.1), did not support growth of *Xf* (not shown). Given the variability of chemical composition of xylem sap due to genotypic, soil and seasonal parameters, more studies on the chemical and physical properties of various saps and media are warranted to better understand their effect on planktonic, sedimented and attached populations of *Xf*. Transcriptome analysis comparing cells in xylem sap and artificial media may also reveal genetic determinants that are essential for *Xf* virulence and have been overlooked thus far. With this in mind, the effect on biofilm formation and cell motility of sap from different *Vitis* species (*Vitis vinifera* cv. Chardonnay, *Vitis labrusca* and *Vitis shuttleworthii*) has been initiated in our laboratory. Other ongoing projects include the study of the effect of sap on motility of wild type and pili-deficient *Xf* mutants in microfluidic chambers, the effect of sap on the expression of genes involved in motility, and also the study of cell viability within biofilms.

In cases where transferring from artificial media directly to xylem sap is lethal or bacteriostatic to *Xf*, a stepwise acclimation using sap : PD2 mixtures starting at 50% sap and moving to 80%, 90% and then 100% sap at each weekly passage is suggested. Although it is possible to culture *Xf* in pure sap, the culture reaches stationary phase faster, probably due to nutrient limitations. Under these conditions passages should be made with a maximum of 3–5 days if 100% sap is being used. If faster growth and greater cell mass is desired, a 9 : 1 sap : PD2 mixture should be used. For experiments involving plant inoculation (by insect or needle), acclimating cells to 90% sap or even 100% sap might increase the number of virulent and viable cells in the plant, accelerating symptom development. Investigations in our laboratory are underway to test this.

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